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(54) Title: CLEAVED AMPLIFIED RFLP DETECTION METHODS

(57) Abstract

The invention features methods for generating and detecting polymorphic restriction sites in nucleic acids, and kits for carrying out these methods.

CLEAVED AMPLIFIED RFLP DETECTION METHODS

Background of the Invention

This invention relates to the generation and detection of genetic polymorphisms.

Genetic maps consisting primarily of restriction fragment length polymorphic (RFLP) markers are being constructed for many organisms, including man. Traditional approaches for detecting RFLPs involve Southern blot hybridization. Recently, techniques based on the polymerase chain reaction (PCR; Mullis et al., Methods Enzymol. 155:350-355, 1987) have been used in addition to, or in place of, traditional RFLP markers in genetic analysis (Cox et al., BioEssays 13:193-198, 1991). In contrast to traditional RFLP markers, PCR-generated markers can be scored using a small sample of DNA, without the use of radioactivity, and without the need for time-consuming DNA blotting procedures.

One widely used PCR-based approach involves the use of single short PCR primers of arbitrary sequence called RAPD primers (for random amplified polymorphic DNA; Reiter et al., Proc. Natl. Acad. Sci. USA 89:1477-1481, 1992; Williams et al., Theoret. Appl. Genet. 82:489-498, 1991). A second category of PCR-based markers are called SSLPs (for simple sequence length polymorphism). The method employing SSLPs is based on amplification across tandem repeats of one or a few nucleotides known as "microsatellites." Microsatellites occur frequently and randomly in most eukaryotic genomes and display a high degree of polymorphism due to variation in the numbers of repeated units.

A third category of PCR-based markers are called AFLPs (for amplified fragment length polymorphisms). In the method employing these markers, DNA from two

polymorphic strains are cleaved with one or two restriction endonucleases, and adapters are ligated to the ends of the cleaved fragments. The fragments are then amplified using primers complementary to the adapter(s). The primers contain short stretches of random nucleotides at their 3′ ends, which result in limiting the number of amplified fragments generated.

Summary of the Invention

We have developed novel PCR-based methods for detecting the presence or absence of a polymorphic restriction site in a nucleic acid involving the use of differentially labeled PCR primers and oligonucleotides.

Accordingly, in one aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a specific binding pair, the second primer being tagged with a detectable label; (b) digesting the PCR product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) contacting the reaction product of step (b) with the second member of the specific binding pair, immobilized on a solid support; and (d) measuring the level of the detectable label bound to the solid support, the presence of the detectable label bound to the solid support being an indication of the absence of the polymorphic restriction site in the nucleic acid.

In a second aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR

using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a specific binding pair, the second primer being tagged with a first detectable label; (b) digesting the PCR product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) annealing and ligating to the single-stranded ends generated in the reaction of step (b) an oligonucleotide tagged with a second detectable label; (d) contacting the reaction product of step (c) with the second member of the specific binding pair, immobilized on a solid support; and (e) determining the levels of the first and second detectable labels bound to the solid support, the presence of only the first detectable label bound to the solid support being an indication of a homozygote lacking the polymorphic restriction site, the presence of only the second detectable label bound to the solid support being an indication of a homozygote containing the polymorphic restriction site, and the presence of both the first and second detectable labels bound to the solid support being an indication of a heterozygote.

In a third aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the method involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled; (b) digesting a portion of the reaction of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site, while leaving another portion of the reaction of step (a) undigested; (c) denaturing the digested and undigested portions from step (b); (d) contacting the product of step (c) with an

oligonucleotide complementary to a sequence in the strand of the product of step (c) containing the detectable label, the sequence being between the polymorphic restriction and the sequence complementary to the second primer, the oligonucleotide being tagged with a first member of a specific binding pair; (e) contacting the reaction product of step (d) with the second member of the specific binding pair, immobilized on a solid support; and (f) determining the ratio of the levels of the detectable label bound to the solid support between undigested and digested samples, a ratio of 1:0 between equivalent portions of the undigested and digested samples being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent portions of the undigested and digested samples being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent portions of the undigested and digested samples being an indication of a heterozygote.

In a fourth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, the second primer being tagged with a second detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) denaturing the reaction product of step (b); (d) contacting the product of step (c) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the product of step (c) containing the first detectable label, the first sequence being between

the polymorphic restriction site and the sequence corresponding to the first primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, the second oligonucleotide being complementary to a second sequence in the strand of the product of step (c) containing the second detectable label, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being complementary to either of the first or second primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (e) contacting a first portion of the reaction product of step (d) with the second member of the first specific binding pair, immobilized on a first solid support; (f) contacting a second portion of the reaction product of step (d) with the second member of the second specific binding pair, immobilized on a second solid support; and (g) determining the ratio of the levels of the first and second detectable labels bound to the first and second solid supports, a ratio of 1:0 between equivalent amounts of the first and second portions being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and second portions being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent amounts of the first and second portions being an indication of a heterozygote.

In a fifth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, the second primer being tagged with a

second detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) denaturing the reaction product of step (b); (d) contacting the product of step (c) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the product of step (c) containing the first detectable label, the first sequence being between the polymorphic restriction site and the sequence complementary to the second primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, the second oligonucleotide being complementary to a second sequence in the strand of the product of step (c) containing the second detectable label, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being complementary to either of the first or second primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (e) contacting a first portion of the reaction product of step (d) with the second member of the first specific binding pair, immobilized on a first solid support; (f) contacting a second portion of the reaction product of step (d) with the second member of the second specific binding pair, immobilized on a second solid support; and (g) determining the ratio of the levels of the first and second detectable labels bound to the first and second solid supports, a ratio of 0:1 between equivalent amounts of the first and second portions being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and second portions being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of

1:2 between equivalent amounts of the first and second portions being an indication of a heterozygote.

In a sixth aspect, the invention method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, the second primer being tagged with a second detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) denaturing the reaction product of step (b); (d) contacting the product of step (c) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the product of step (c) containing the first detectable label, the first sequence being between the polymorphic restriction site and the sequence corresponding to the first primer, the first oligonucleotide being tagged with the first member of a specific binding pair, the second oligonucleotide being complementary to a second sequence in the strand of the product of step (c) containing the second detectable label, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being complementary to either of the first or second primers, the second oligonucleotide being tagged with the first member of the specific binding pair; (e) contacting the reaction product of step (d) with the second member of the specific binding pair, immobilized on a solid support; and (f) determining the ratio of the levels of the first and second detectable labels bound to the solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site,

a ratio of 1:1 being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 being an indication of a heterozygote.

In a seventh aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, the second primer being tagged with a second detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) denaturing the reaction product of step (b); (d) contacting the product of step (c) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the product of step (c) containing the first detectable label, the first sequence being between the polymorphic restriction site and the sequence complementary to the second primer, the first oligonucleotide being tagged with the first member of a specific binding pair, the second oligonucleotide being complementary to a second sequence in the strand of the product of step (c) containing the second detectable label, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being complementary to either of the first or second primers, the second oligonucleotide being tagged with the first member of the specific binding pair; (e) contacting the reaction product of step (d) with the second member of the specific binding pair, immobilized on a solid support; and (f) determining the ratio of the levels of the first and second detectable labels bound to the solid

support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:2 being an indication of a heterozygote.

In an eighth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) contacting the reaction product of step (b) with the second member of the first specific binding pair, immobilized on a first solid support; (d) denaturing the reaction product of step (c) not bound to the first solid support; (e) contacting the product of step (d) with an oligonucleotide complementary to a sequence in the strand of the product of step (d) containing the detectable label, the sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being tagged with the first member of a second specific binding pair; (f) contacting the reaction product of step (e) with the second member of the second specific binding pair, immobilized on a second solid support; and (g) determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step

(c) not bound to the first solid support was used in steps (d), (e), and (f); a ratio of 1:0 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d), (e), and (f); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d), (e), and (f). In a ninth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) annealing and ligating to the single-stranded ends generated in the reaction of step (b) a first oligonucleotide tagged with the first member of a first specific binding pair; (d) contacting the reaction product of step (c) with the second member of the first specific binding pair, immobilized on a first solid support; (e) denaturing the reaction product of step (d) not bound to the first solid support; (f) contacting the product of step (e) with a second oligonucleotide complementary to a sequence in the strand of the product of step (e) containing the detectable label, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the second oligonucleotide being tagged with the first member of a second specific binding pair; (g) contacting the reaction product of step (f) with the

second member of the second specific binding pair, immobilized on a second solid support; and (h) determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g); a ratio of 0:1 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g); and a ratio of 1:1 being an indication of a heterozygote; in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g).

In a tenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) contacting the reaction product of step (b) with the second member of the first specific binding pair, immobilized on a first solid support; (d) denaturing the reaction product from step (c) not bound to the first solid support; (e) contacting the product of step (d) with an oligonucleotide complementary to a sequence in the strand of the product of step (d)

containing the detectable label, the sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being immobilized on a second solid support; and (f) determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d) and (e); a ratio of 1:0 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d) and (e); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (c) not bound to the first solid support was used in steps (d) and (e).

In an eleventh aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled; (b) digesting the reaction product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site; (c) annealing and ligating to the single-stranded ends generated in the reaction of step (b) a first oligonucleotide tagged with the first member of a first specific binding pair; (d) contacting the reaction product of step (c) with the second member of the first specific binding pair, immobilized on a first solid support; (e) denaturing the reaction product

of step (d) not bound to the first solid support; (f) contacting the product of step (e) with a second oligonucleotide complementary to a sequence in the strand of the product of step (e) containing the detectable label, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the second oligonucleotide being immobilized on a second solid support; and (g) determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f); a ratio of 0:1 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f).

In a twelfth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing

the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) digesting the product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) contacting the reaction product of step (c) with the second member of the specific binding pair, immobilized on a solid support; and (e) measuring the level of the detectable label bound to the solid support, the presence of the detectable label bound to the solid support being an indication of the absence of the polymorphic restriction site in the nucleic acid.

In a thirteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) digesting the PCR product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) annealing and ligating to the single-stranded ends

generated in the reaction of step (c) an oligonucleotide tagged with a second detectable label; (e) contacting the reaction product of step (d) with the second member of the specific binding pair, immobilized on a solid support; and (f) determining the levels of the first and second detectable labels bound to the solid support, the presence of only the first detectable label bound to the solid support being an indication of a homozygote lacking the polymorphic restriction site, the presence of only the second detectable label bound to the solid support being an indication of a homozygote containing the polymorphic restriction site, and the presence of both the first and second detectable labels bound to the solid support being an indication of a heterozygote.

In a fourteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third primer and the second primer, the third primer containing the first sequence, the third primer being tagged with a detectable label; (c) digesting a portion of the reaction of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site, while leaving another portion of the reaction of step (b) undigested; (d) denaturing the digested and undigested portions from step (c); (e) contacting the product of step (d) with an oligonucleotide complementary to a second sequence in the strand of the product of step (d) containing the detectable label, the second sequence being between the polymorphic restriction site and the sequence complementary to the second primer, the

oligonucleotide being tagged with a first member of a specific binding pair; (f) contacting the reaction product of step (e) with the second member of the specific binding pair, immobilized on a solid support; and (g) determining the ratio of the levels of the detectable label bound to the solid support between undigested and digested samples, a ratio of 1:0 between equivalent portions of the undigested and digested samples being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent portions of the undigested and digested samples being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent portions of the undigested and digested samples being an indication of a heterozygote.

In a fifteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a second detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) denaturing the reaction product of step (c); (e) contacting the product

of step (d) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a third sequence in the strand of the product of step (d) containing the first detectable label, the third sequence being between the polymorphic restriction site and the sequence corresponding to or complementary to the first primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, the second oligonucleotide being complementary to a fourth sequence in the strand of the product of step (d) containing the second detectable label, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (f) contacting a first portion of the reaction product of step (e) with the second member of the first specific binding pair, immobilized on a first solid support; (g) contacting a second portion of the reaction product of step (e) with the second member of the second specific binding pair, immobilized on a second solid support; and (h) determining the ratio of the levels of the first and second detectable labels bound to the first and second solid supports, a ratio of 1:0 between equivalent amounts of the first and second portions being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and second portions being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent amounts of the first and second portions being an indication of a heterozygote.

In a sixteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving

the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a second detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) denaturing the reaction product of step (c); (e) contacting the product of step (d) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a third sequence in the strand of the product of step (d) containing the first detectable label, the third sequence being between the polymorphic restriction site and the sequence corresponding to or complementary to the second primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, the second oligonucleotide being complementary to a fourth sequence in the strand of the product of step (d) containing the second detectable label, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (f) contacting a first portion of the reaction product of step (e) with the second member of the first specific binding pair,

immobilized on a first solid support; (g) contacting a second portion of the reaction product of step (e) with the second member of the second specific binding pair, immobilized on a second solid support; and (h) determining the ratio of the levels of the first and second detectable labels bound to the first and second solid supports, a ratio of 0:1 between equivalent amounts of the first and second portions being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and second portions being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:2 between equivalent amounts of the first and second portions being an indication of a heterozygote.

In a seventeenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a second detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) denaturing the reaction product of step (c); (e) contacting the product of step (d) with a first and a second oligonucleotide,

the first oligonucleotide being complementary to a third sequence in the strand of the product of step (d) containing the first detectable label, the third sequence being between the polymorphic restriction site and the sequence corresponding to or complementary to the first primer, the first oligonucleotide being tagged with the first member of a specific binding pair, the second oligonucleotide being complementary to a fourth sequence in the strand of the product of step (d) containing the second detectable label, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member of the specific binding pair; (f) contacting the reaction product of step (e) with the second member of the specific binding pair, immobilized on a solid support; and (g) determining the ratio of the levels of the first and second detectable labels bound to the solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 being an indication of a heterozygote.

In an eighteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving (a) amplifying the nucleic acid by PCR using a first and second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the

first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a second detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) denaturing the reaction product of step (c); (e) contacting the product of step (d) with a first and a second oligonucleotide, the first oligonucleotide being complementary to a third sequence in the strand of the product of step (d) containing the first detectable label, the third sequence being between the polymorphic restriction site and the sequence corresponding to or complementary to the second primer, the first oligonucleotide being tagged with the first member of a specific binding pair, the second oligonucleotide being complementary to a fourth sequence in the strand of the product of step (d) containing the second detectable label, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member of the specific binding pair; (f) contacting the reaction product of step (e) with the second member of the specific binding pair, immobilized on a solid support; and (g) determining the ratio of the levels of the first and second detectable labels bound to the solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 being an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:2 being an indication of a heterozygote.

In a nineteenth aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a first specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) contacting the reaction product of step (c) with the second member of the first specific binding pair, immobilized on a first solid support; (e) denaturing the reaction product of step (d) not bound to the first solid support; (f) contacting the product of step (e) with an oligonucleotide complementary to a third sequence in the strand of the product of step (e) containing the detectable label, the third sequence being between the polymorphic restriction site and the sequence corresponding to or complementary to the second primer, the oligonucleotide being tagged with the first member of a second specific binding pair; (g) contacting the reaction product of step (f) with the second member of the second specific binding pair, immobilized on a second solid support; and (h) determining the ratio of the level of the detectable label bound to the first solid support

to the level of the detectable label bound to the second solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g); a ratio of 1:0 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e), (f), and (g).

In a twentieth aspect the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third primer and the second primer, the third primer containing the first sequence, the third primer being tagged with a detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) annealing and ligating to the single-stranded ends generated in the reaction of step (c) a first oligonucleotide tagged with the first member of a first specific binding pair; (e) contacting the reaction product of step (d) with the second member of the first specific binding pair, immobilized on a first solid support; (f) denaturing the reaction product of step (e) not bound to the first solid support; (g) contacting the

product of step (f) with a second oligonucleotide complementary to a second sequence in the strand of the product of step (f) containing the detectable label, the second sequence being between the polymorphic restriction site and either the sequence corresponding to or complementary to the second primer or the sequence corresponding to or complementary to the first primer, the second oligonucleotide being tagged with the first member of a second specific binding pair; (h) contacting the reaction product of step (g) with the second member of the second specific binding pair, immobilized on a second solid support; and (i) determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f), (g), and (h); a ratio of 0:1 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f), (g), and (h); and a ratio of 1:1 being an indication of a heterozygote; in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f), (g), and (h).

In a twenty-first aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second

sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a first specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) contacting the reaction product of step (c) with the second member of the first specific binding pair, immobilized on a first solid support; (e) denaturing the reaction product from step (d) not bound to the first solid support; (f) contacting the product of step (e) with an oligonucleotide complementary to a third sequence in the strand of the product of step (e) containing the detectable label, the third sequence being between the polymorphic restriction site and the sequence corresponding to or complementary to the second primer, the oligonucleotide being immobilized on a second solid support; and (g) determining the ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support, a ratio of 0:1 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f); a ratio of 1:0 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f); and a ratio of 1:1 being an indication of a heterozygote, in a case

where the total amount of the reaction product from step (d) not bound to the first solid support was used in steps (e) and (f).

In a twenty-second aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the method involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) amplifying the product of step (a) by PCR using a third primer and the second primer, the third primer containing the first sequence, the third primer being tagged with a detectable label; (c) digesting the reaction product of step (b) with the restriction endonuclease corresponding to the polymorphic restriction site; (d) annealing and ligating to the single-stranded ends generated in the reaction of step (c) a first oligonucleotide tagged with the first member of a first specific binding pair; (e) contacting the reaction product of step (d) with the second member of the first specific binding pair, immobilized on a first solid support; (f) denaturing the reaction product of step (e) not bound to the first solid support; (g) contacting the product of step (f) with a second oligonucleotide complementary to a second sequence in the strand of the product of step (f) containing the detectable label, the second sequence being between the polymorphic restriction site and either the sequence corresponding to or complementary to the second primer or the sequence corresponding to or complementary to the first primer, the second oligonucleotide being immobilized on a second solid support; and (h) determining the ratio of the level of the detectable label bound to the first solid support to the level of

the detectable label bound to the second solid support, a ratio of 1:0 being an indication of a homozygote containing the polymorphic restriction site, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f) and (g); a ratio of 0:1 being an indication of a homozygote lacking the polymorphic restriction site, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f) and (g); and a ratio of 1:1 being an indication of a heterozygote, in a case where the total amount of the reaction product from step (e) not bound to the first solid support was used in steps (f) and (g).

In a twenty-third aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing one or more sets of a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a specific binding pair, the second primer being tagged with a detectable label. In a preferred embodiment, the kit further contains the second member of the specific binding pair, immobilized on a solid support. In another preferred embodiment, the kit further contains an oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide being tagged with a second detectable label.

In a twenty-fourth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being

unlabeled; (b) an oligonucleotide complementary to a sequence in the strand of the nucleic acid complementary to the second primer, the sequence being between the polymorphic restriction site and the sequence complementary to the second primer, the oligonucleotide being tagged with a first member of a specific binding pair; and (c) the second member of the specific binding pair, immobilized on a solid support.

In a twenty-fifth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, the second primer being tagged with a second detectable label; (b) a first oligonucleotide, the first oligonucleotide being complementary to a first sequence in the strand of the nucleic acid complementary to the second primer, the first sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the first oligonucleotide being tagged with the first member of a first specific binding pair; (c) a second oligonucleotide, the second oligonucleotide being complementary to a second sequence in the strand of the nucleic acid complementary to the first primer, the second sequence being on the same side of the polymorphic restriction site as the first sequence, the second sequence not being contained within or being complementary to either of the first or second primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (d) the second member of the first specific binding pair, immobilized on a first solid support; and (e) the second member of the second specific binding pair, immobilized on a second

solid support. In a preferred embodiment, the first and the second specific binding pairs are identical, and the first and the second solid supports are identical.

In a twenty-sixth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label; (b) the second member of the first specific binding pair, immobilized on a first solid support; (c) an oligonucleotide complementary to a first sequence in the strand of the nucleic acid containing the sequence corresponding to the second primer, the first sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being tagged with the first member of a second specific binding pair; and (d) the second member of the second specific binding pair, immobilized on a second solid support.

In a twenty-seventh aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled; (b) a first oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide being tagged with the first member of a first specific binding pair; (c) the second member of the first specific binding pair, immobilized on a first solid support; (d) a second oligonucleotide complementary

to a sequence in the strand of the nucleic acid complementary to the second primer, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the second oligonucleotide being tagged with the first member of a second specific binding pair; and (e) the second member of the second specific binding pair, immobilized on a second solid support.

In a twenty-eighth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label; (b) the second member of the first specific binding pair, immobilized on a first solid support; and (c) an oligonucleotide complementary to a first sequence in the strand of the nucleic acid containing the sequence corresponding to the second primer, the first sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being immobilized on a second solid support.

In a twenty-ninth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled; (b) a first oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site,

the oligonucleotide being tagged with the first member of a first specific binding pair; (c) the second member of the first specific binding pair, immobilized on a first solid support; and (d) a second oligonucleotide complementary to a sequence in the strand of the nucleic acid complementary to the second primer, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, the second oligonucleotide being immobilized on a second solid support.

In a thirtieth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label. In a preferred embodiment, the kit further contains the second member of the specific binding pair, immobilized on a solid support. In another preferred embodiment, the kit further contains an oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide being tagged with a second detectable label. In a thirty-first aspect, the invention

features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) a third primer containing the first sequence, the third primer being tagged with a detectable label; (c) an oligonucleotide complementary to a second sequence in the strand of the nucleic acid containing the sequence complementary to the second primer, the second sequence being between the polymorphic restriction site and the sequence complementary to the second primer, the oligonucleotide being tagged with a first member of a specific binding pair; and (d) the second member of the specific binding pair, immobilized on a solid support.

In a thirty-second aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with a first detectable label, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a second detectable label; (c) a first oligonucleotide, the first oligonucleotide being complementary to a third sequence in the strand of the nucleic acid complementary to the second primer, the third sequence being between the polymorphic restriction site and either the sequence

complementary to the second primer or the sequence corresponding to the first primer, the first oligonucleotide being tagged with the first member of a first specific binding pair, (d) a second oligonucleotide, the second oligonucleotide being complementary to a fourth sequence in the strand of the nucleic acid complementary to the first primer, the fourth sequence being on the same side of the polymorphic restriction site as the third sequence, the fourth sequence not being contained within or being complementary to any of the primers, the second oligonucleotide being tagged with the first member of a second specific binding pair; (e) the second member of the first specific binding pair, immobilized on a first solid support; and (f) the second member of the second specific binding pair, immobilized on a second solid support. In a preferred embodiment, the first and the second specific binding pairs are identical, and the first and the second solid supports are identical.

In a thirty-third aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a first specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) the second member of the first

specific binding pair, immobilized on a first solid support; (d) an oligonucleotide complementary to a third sequence in the strand of the nucleic acid corresponding to the second primer, the sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being tagged with the first member of a second specific binding pair; and (e) the second member of the second specific binding pair, immobilized on a second solid support.

In a thirty-fourth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) a third primer containing the first sequence, the third primer being tagged with a detectable label; (c) a first oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide being tagged with the first member of a first specific binding pair; (d) the second member of the first specific binding pair, immobilized on a first solid support; (e) a second oligonucleotide complementary to a second sequence in the strand of the nucleic acid corresponding to the first primer, the second sequence being between the polymorphic restriction site and either the sequence complementary to the second primer or the sequence corresponding to the first primer, the second oligonucleotide being tagged with the first member of a second specific binding pair; and (f) the second member

of the second specific binding pair, immobilized on a second solid support.

In a thirty-fifth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid, the second primer containing a second sequence not complementary to or present in the nucleic acid; (b) a third and a fourth primer, the third primer containing the first sequence or a sequence complementary to the first sequence, the third primer being tagged with the first member of a first specific binding pair, the fourth primer containing the second sequence or a sequence complementary to the second sequence, the fourth primer being tagged with a detectable label; (c) the second member of the first specific binding pair, immobilized on a first solid support; and (d) an oligonucleotide complementary to a third sequence in the strand of the nucleic acid corresponding to the second primer, the third sequence being between the polymorphic restriction site and the sequence corresponding to the second primer, the oligonucleotide being immobilized on a second solid support.

In a thirty-sixth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: (a) a first and a second primer flanking the polymorphic restriction site, the first primer containing a first sequence not complementary to or present in the nucleic acid; (b) a third primer containing the first sequence, the third primer being tagged with a detectable label; (c) a first

oligonucleotide complementary to the single-stranded ends generated in the nucleic acid upon digestion of the nucleic acid with the restriction enzyme corresponding to the polymorphic restriction site, the oligonucleotide being tagged with the first member of a first specific binding pair; (d) the second member of the first specific binding pair, immobilized on a first solid support; and (e) a second oligonucleotide complementary to a second sequence in the strand of the nucleic acid corresponding to the first primer, the sequence being between the polymorphic restriction site and either the sequence corresponding to or complementary to the second primer or the sequence corresponding to or complementary to the first primer, the second oligonucleotide being immobilized on a second solid support.

In a thirty-seventh aspect, the invention features a method for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, involving the steps of: (a) amplifying the nucleic acid by PCR using a first and a second primer flanking the polymorphic restriction site, whereby the resultant PCR product is of a defined size readily resolved by gel electrophoresis; (b) digesting the PCR product of step (a) with the restriction endonuclease corresponding to the polymorphic restriction site, the digestion products being differentially sized; (c) separating the reaction products of step (b) by gel electrophoresis; and (d) detecting the separated reaction products, the presence of only uncleaved products being an indication of a homozygote lacking the polymorphic restriction site, the presence of only cleaved products being an indication of a homozygote containing the polymorphic restriction site, and the presence of both cleaved and uncleaved products being an indication of a heterozygote. In a preferred embodiment, one or both of the first and second

primers are tagged with a detectable label. In another preferred embodiment, the PCR product is 100-1000 base pairs in length.

In a thirty-eighth aspect, the invention features a kit for detecting the presence or absence of a polymorphic restriction site in a nucleic acid, the kit containing: a first and a second primer flanking the polymorphic restriction site and capable of generating a PCR product of a defined size that is readily resolved by gel electrophoresis. In a preferred embodiment, the first and/or the second primers are detectably labeled. In another preferred embodiment, the PCR product generated is between 100 and 1000 base pairs in length.

In a thirty-ninth aspect, the invention features a method for identifying a polymorphic restriction site in a nucleic acid, involving the steps of: (a) digesting DNA isolated from a first sample with a first restriction endonuclease; (b) ligating to each of the ends of the reaction products of step (a) a first adaptor; (c) digesting the products of step (b) with a second restriction endonuclease; (d) ligating to each of the ends of the reaction products generated in step (c) a second adaptor; (e) amplifying the reaction products of step (d) by PCR using a first primer complementary to the first adaptor and a second primer complementary to the second adaptor, the second primer being tagged with a first member of a specific binding pair (preferably, biotin); (f) in a separate set of reactions, digesting DNA isolated from a second sample with the first restriction endonuclease; (g) ligating to each of the ends of the reaction products of step (f) a third adaptor; (h) digesting the products of step (g) with the second restriction endonuclease; (i) denaturing the products of step (e) and the products of step (h); (j) combining the denatured products of step (i) under

conditions allowing hybridization; (k) contacting the hybridization products of step (j) with the second member of the specific binding pair (preferably, avidin), the second member being immobilized on a solid support; (l) recovering the hybridization products captured on the solid support; and (m) amplifying the products obtained in step (l) by PCR using a primer complementary to the third adaptor, an amplified product being an indication of a polymorphic restriction site corresponding to the second restriction endonuclease.

In a related aspect, the invention features a kit for identifying a polymorphic restriction site in a nucleic acid, the kit comprising: (a) a first DNA adaptor, a second DNA adaptor, and a third DNA adaptor, the first and third DNA adaptors having regions complementary to the ends generated by a first restriction endonuclease ends but differing in overall sequence and the second DNA adaptor having a region complementary to the ends generated by a second restriction endonuclease, the second restriction endonuclease site corresponding to the polymorphic restriction site; and (b) a first primer, a second primer, and a third primer, the first primer being complementary to the first DNA adaptor, the second primer being complementary to the second DNA adaptor and being tagged with a first member of a specific binding pair, and the third primer being complementary to the third DNA adaptor. This kit may further comprises the second member of the specific binding pair immobilized on a solid support.

In a preferred embodiment of various of the above aspects, multiple polymorphic restriction sites are detected by the method or kit. In preferred embodiments of various of the above aspects, the detectable label is selected from the group consisting of digoxigenin,

fluorescent labels (e.g., fluorescein and rhodamine), enzymes (e.g., horseradish peroxidase and alkaline phosphatase), biotin (which can be detected by anti-biotin specific antibodies or enzyme-conjugated avidin derivatives), radioactive labels (e.g., ^{32}P and ^{125}I), colorimetric reagents, and chemiluminescent reagents.

In other preferred embodiments of various of the above aspects, the specific binding pairs are selected from the group consisting of avidin-biotin, streptavidin-biotin, hybridizing nucleic acid pairs, interacting protein pairs, antibody-antigen pairs, reagents containing chemically reactive groups (e.g., reactive amino groups), and nucleic acid sequence-nucleic acid binding protein pairs. In related preferred embodiments of various of the above aspects, the solid supports used in the methods of the invention are selected from the group consisting of agarose, acrylamide, or polystyrene beads; polystyrene microtiter plates (for use in, e.g., ELISA); and nylon and nitrocellulose membranes (for use in, e.g., dot or slot blot assays).

The term "heterozygote," as used herein, refers to an individual with different alleles at corresponding loci on homologous chromosomes. Accordingly, the term "heterozygous," as used herein, describes an individual or strain having different allelic genes at one or more paired loci on homologous chromosomes.

The term "homozygote," as used herein, refers to an individual with the same allele at corresponding loci on homologous chromosomes. Accordingly, the term "homozygous," as used herein, describes an individual or a strain having identical allelic genes at one or more paired loci on homologous chromosomes.

The term "corresponding" as used herein to describe a nucleic acid strand, e.g., a nucleic acid strand corresponding to a particular PCR primer, is meant

to indicate that the strand contains the sequence of the particular PCR primer. When used to compare a polymorphic restriction site to a restriction endonuclease site, the term again indicates that the two sequences are identical.

An advantage of certain detection methods of the present invention over many other methods used to detect genetic polymorphisms is that gel electrophoresis is not required in the analysis. Thus, the methods of the present invention are readily adaptable for automation, allowing large numbers of samples to be processed in relatively short periods of time, at lower costs. In addition, in several variations of the methods of the invention (see, e.g., Examples III and IV below), internal controls are provided, thus controlling for variability detected by different practitioners. Furthermore, in several of the variations of the methods of the invention (see Examples III - VIII below), an oligonucleotide probe hybridizing to a sequence in the PCR product internal to the primers is used to purify the products, thus allowing a reduction in background problems associated with PCR amplification.

Those detection methods of the invention utilizing gel electrophoresis are also advantageous because they provide a rapid and inexpensive approach to the identification of large numbers of PCR-based genetic and RFLP markers.

The method of the invention useful for cloning genetic polymorphisms also represents an improvement over current methods. Because the process of selecting out a tagged (e.g., biotinylated) DNA having a polymorphism involves a specific hybridization step, candidate DNA from any source may be utilized. For example, DNA from random clones, cDNA libraries, YAC libraries, or any other DNA collection may be screened; pure preparations

of genomic DNAs are not required. Moreover, like other methods of the invention, this cloning procedure is rapid and inexpensive.

All methods of the invention are useful in clinical diagnostic testing, genomic mapping, positional cloning of genes defined by mutation (such as those that cause inherited disease in humans or resistance to pathogens in crop plants), DNA fingerprinting (e.g., for forensic analysis and paternity testing), crop and livestock breeding programs, and other related applications.

In one particular example, the detection methods of the invention are useful for bacterial typing utilizing known conserved polymorphic sequences diagnostic of the bacterium. In one application, this approach is useful for distinguishing one bacterium from another (e.g., for the identification of Salmonella in a food sample); polymorphism-containing sequences preferred for this approach include those present in conserved ribosomal RNA genes. In another application, this approach is useful for screening bacteria (e.g., clinical isolates) for antibiotic resistance; in this case, known polymorphic restriction sites within the antibiotic resistance marker are utilized. The instant methods of bacterial typing decrease false positive results frequently obtained using current PCR-based techniques.

Detailed Description

The drawings are first described.

Drawings

Fig. 1 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a detectable label (X) and a second PCR primer tagged with the first member of a specific binding pair (Y). After amplification by PCR, the products are digested with the

restriction endonuclease (R) corresponding to the polymorphic restriction site; contacted with the second member of the specific binding pair immobilized on a solid support, and the level of the detectable label (X) bound to the solid support is determined.

Fig. 2 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a first detectable label (X) and a second PCR primer tagged with the first member of a specific binding pair (Y). After amplification by PCR, the products are digested with the restriction endonuclease (R) corresponding to the polymorphic restriction site, and an oligonucleotide tagged with a second detectable label (Z) is annealed and ligated to the single-stranded ends generated in the digestion. The reaction is then contacted with the second member of the specific binding pair bound to a solid support, and the levels of the first and second detectable labels (X and Z) bound to the solid support are determined.

Fig. 3 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a detectable label (P1) and a second unlabeled PCR primer (P2). After amplification by PCR, half of the reaction (or one of the identical reactions if carried out in duplicate) is digested with the restriction endonuclease (R) corresponding to the polymorphic restriction site. Both digested and undigested reactions are then denatured and contacted with an oligonucleotide tagged with the first member of a specific binding pair, the oligonucleotide being complementary to the P1 strand and located to the right of the restriction site (R) near to, but not overlapping, primer P2. The reactions are then contacted with the second member of the specific binding pair immobilized on a solid support, and the levels of P1 in digested versus undigested reactions are compared.

Fig. 4 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a first detectable label (P1) and a second PCR primer tagged with a second detectable label (P2). After amplification by PCR, the products are digested with the restriction endonuclease (R) corresponding to the polymorphic restriction site, denatured, and contacted with a first oligonucleotide complementary to the P1 strand and located to the right of the restriction site (R) near to, but not overlapping primer P2, and a second oligonucleotide complementary to the P2 strand and located to the right of the restriction site (R) near to, but not overlapping the sequence complementary to primer P2. Both the first and second oligonucleotides are tagged with the first member of a specific binding pair (Y). The reactions are then contacted with the second member of the specific binding pair immobilized on a solid support, and the ratio of P1 to P2 bound to the solid support is determined.

Fig. 5 is a schematic of a RFLP detection method involving the use of a first PCR primer tagged with a detectable label (X) and a second PCR primer tagged with the first member of a first specific binding pair (Y). After amplification by PCR, the products are digested with the restriction enzyme (R) corresponding to the polymorphic restriction site, and are contacted with the second member of the first specific binding pair immobilized on a first solid support. The filtrate is then bound to a solid support with the anchor sequence (or contacted with an oligonucleotide complementary to the X strand between the restriction site (R) and the label (X), the oligonucleotide being tagged with the first member of a second specific binding pair, and then contacted with the second member of the second specific binding pair immobilized on a second solid support), and

the levels of the detectable label bound to the first solid support and the anchor sequence (or second solid support) are determined.

Fig. 6 is a schematic of a RFLP detection method involving the use of a first unlabeled PCR primer and a second PCR primer tagged with a detectable label (X). After amplification by PCR, the products are digested with the restriction enzyme (R) corresponding to the polymorphic restriction site, and contacted with an oligonucleotide complementary to the single-stranded ends generated in the digestion, the oligonucleotide being tagged with the first member of a specific binding pair. The products are then contacted with the second member of the first specific binding pair, bound to a first solid support. The filtrate is then bound to a solid support with the anchor sequence (or contacted with an oligonucleotide complementary to the X strand, the oligonucleotide being tagged with the first member of a second specific binding pair, and then contacted with the second member of the second specific binding pair immobilized on a second solid support), and the levels of the detectable label bound to the first solid support and the anchor sequence (or second solid support) are determined.

Fig. 7 is a schematic of a RFLP detection method involving the use of PCR primers flanking the polymorphic restriction site (the "Alu I" site). Following PCR amplification, the reaction products are digested with the restriction endonuclease corresponding to the polymorphic restriction site (Alu I), and the fragments are run on an agarose gel. The separated fragments are detected as an indication of the presence or absence of the polymorphic marker.

Fig. 8 is a schematic of a typical gel analysis according the method described in Fig. 7.

Fig. 9 is a schematic of a method for cloning polymorphic restriction fragments.

Methods for generating and detecting genetic polymorphisms

The present invention provides several methods for detecting Cleaved Amplified Polymorphic Sequences (CAPS; Konieczny et al., The Plant Journal 4(2):403-410, 1993). In the CAPS method, a nucleic acid containing a polymorphic restriction site is amplified using primers flanking the restriction site. The resulting PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and the digested products are analyzed by gel electrophoresis.

The detection methods of the present invention vary greatly from one another in detail, however they share three central features: (1) the nucleic acid containing the polymorphic restriction site is amplified by PCR using differently labeled primers flanking the polymorphic restriction site, (2) the resulting PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site (which will cleave the DNA of some individuals but not cleave the DNA of others, depending on the presence of the polymorphism), and (3) the resulting digestion products are analyzed by detection of the labels they contain, and/or labels attached to oligonucleotides complementary to the digestion products, in order to determine the identity of the polymorphic restriction site. The methods of the invention allow rapid and efficient analyses of a large number of samples.

The nucleic acid sample containing the polymorphic restriction site being analyzed can be obtained from any source, e.g., a tissue homogenate, blood, amniotic fluid,

and chorionic villus samples; and can be obtained from these sources using standard methods. Only a minute quantity of nucleic acid is required, and can be DNA or RNA (in the case of RNA, a reverse transcription step is required before the PCR step). The polymerase chain reactions (PCR) used in the methods of the present invention are carried out using standard methods (see, e.g., Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1989; Erlich, PCR Technology, Stockton Press, New York, 1989; Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, Harcourt Brace Javanovich, New York, 1990; Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989). Restriction enzyme digestion is also carried out by standard methods using any of a number of available restriction endonucleases (see, e.g., Ausubel et al., *supra*; New England Biolabs, Beverly, MA).

The primers and oligonucleotides used in the methods of the present invention are preferably DNA, and can be synthesized using standard techniques and, when appropriate, detectably labeled using standard methods (Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1989). Detectable labels that can be used to tag the primers and oligonucleotides used in the methods of the invention include, but are not limited to, digoxigenin, fluorescent labels (e.g., fluorescein and rhodamine), enzymes (e.g., horseradish peroxidase and alkaline phosphatase), biotin (which can be detected by anti-biotin specific antibodies or enzyme-conjugated avidin derivatives), radioactive labels (e.g., ^{32}P and ^{125}I), colorimetric reagents, and chemiluminescent reagents. The labels used in the methods of the invention are detected using standard methods.

The specific binding pairs useful in the methods of the invention include, but are not limited to, avidin-biotin, streptavidin-biotin, hybridizing nucleic acid pairs, interacting protein pairs, antibody-antigen pairs, reagents containing chemically reactive groups (e.g., reactive amino groups), and nucleic acid sequence-nucleic acid binding protein pairs.

The solid supports useful in the methods of the invention include, but are not limited to, agarose, acrylamide, or polystyrene beads; polystyrene microtiter plates (for use in, e.g., ELISA); and nylon and nitrocellulose membranes (for use in, e.g., dot or slot blot assays).

The methods of the invention can be facilitated by the use of kits which contain the reagents required for carrying out the assays. The kits can contain reagents for carrying out the generation or analysis of a single polymorphic restriction site (for use in, e.g., diagnostic methods), or multiple polymorphic restriction sites (for use in, e.g., genomic mapping). When multiple samples are analyzed, multiple sets of the appropriate primers and oligonucleotides are provided in the kit. In addition to the primers and oligonucleotides required for carrying out the various methods, the kits may contain the enzymes used in the methods, and the reagents for detecting the labels, e.g., the substrates for enzyme labels, etc.

As discussed above, the invention provides methods and kits for generating and detecting the presence or absence of a polymorphic restriction site in a nucleic acid. Examples I-IX describe eight variations of the methods of the invention. Example X describes a preferred use for the methods of the invention. Example XI describes a preferred method for cloning polymorphic restriction fragments. The following examples are meant

to illustrate, but not limit, the methods of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters of molecular biology which are obvious to those skilled in the art are within the spirit and scope of the present invention.

EXAMPLES

Example I.

In this method, the nucleic acid containing the polymorphism is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a specific binding pair, the second primer being tagged with a detectable label. The resulting PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site and the digested products are contacted with the second member of the specific binding pair, immobilized on a solid support. The level of the detectable label bound to the solid support is then measured. The presence of the detectable label bound to the solid support is an indication of the absence of the polymorphic restriction site in the nucleic acid, while the absence of the detectable label bound to the solid support is an indication of the presence of the polymorphic restriction site in the nucleic acid. An embodiment of this method is shown in Fig. 1.

Example II.

This method is identical to that described in Example I, with the added step of annealing and ligating to the single-stranded ends generated in the digestion reaction, an oligonucleotide tagged with a second detectable label. After applying the reaction to the second member of the specific binding pair, the levels of

both the first and the second detectable labels bound to the solid support are determined. The presence of only the first detectable label bound to the solid support is an indication of a homozygote lacking the polymorphic restriction site, the presence of only the second detectable label bound to the solid support is an indication of a homozygote containing the polymorphic restriction site, and the presence of both the first and the second detectable labels bound to the solid support is an indication of a heterozygote. An embodiment of this method is shown in Fig. 2.

Example III.

In this method, the nucleic acid is amplified using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled. A portion of the PCR reaction is digested with the restriction endonuclease corresponding to the polymorphic restriction site, while another portion is left undigested. Both the digested and undigested portions are then denatured, and contacted with an oligonucleotide tagged with the first member of a specific binding pair. The oligonucleotide is complementary to a sequence in the strand of the PCR product containing the detectable label, the sequence being between the polymorphic restriction site and the sequence complementary to the second primer.

The reaction is then contacted with the second member of the specific binding pair, immobilized on a solid support, and the ratio of the levels of the detectable label bound to the solid support between undigested and digested samples is determined. A ratio of 1:0 between equivalent portions of undigested and digested samples is an indication of a homozygote

containing the polymorphic restriction site, a ratio of 1:1 between equivalent portions of undigested and digested samples is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent portions of undigested and digested samples is an indication of a heterozygote. While the sample volumes used for detection and comparison need not be equivalent, the appropriate calculations must be carried out to account for this adjustment prior to determining the ratio of detectable label in digested and undigested samples. An embodiment of this method is shown in Fig. 3.

Example IV.

In this method, the nucleic acid is amplified by PCR using a first primer and a second primer flanking the polymorphic restriction site, the first primer being tagged with a first detectable label, and the second primer being tagged with a second detectable label.

The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, denatured, and contacting with a first and a second oligonucleotide. The first oligonucleotide is complementary to a first sequence in the strand of the PCR product containing the first detectable label, the first sequence being between the polymorphic restriction site and the sequence corresponding to the first primer. The first oligonucleotide is tagged with the first member of a first specific binding pair. The second oligonucleotide is complementary to a second sequence in the strand of the PCR product containing the second detectable label. The second sequence is on the same side of the polymorphic restriction site as the first sequence, and is not contained within, or complementary to, either the first or the second primer. The second

oligonucleotide is tagged with the first member of a second specific binding pair.

A first portion of the reaction is then contacted with the second member of the first specific binding pair, immobilized on a first solid support, while a second portion of the reaction is contacted with the second member of the second specific binding pair, immobilized on a second solid support. The ratio of the levels of the first and second detectable labels bound to the first and second solid supports is then determined. A ratio of 1:0 between equivalent amounts of the first and second portions is an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:1 between equivalent amounts of the first and second portions is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 2:1 between equivalent amounts of the first and second portions is an indication of a heterozygote.

In the case where the first sequence (to which the first oligonucleotide is complementary) in the strand containing the first detectable label is between the polymorphic restriction site and the sequence complementary to the second primer, the ratios differ, as follows. The ratio of the levels of the first and second detectable labels bound to the first and second solid supports is 0:1 between equivalent amounts of the first and second portions in the case of a homozygote containing the polymorphic restriction site. The ratio is 1:1 between equivalent amounts of the first and second portions in the case of a homozygote lacking the polymorphic restriction site, and the ratio is 1:2 between equivalent amounts of the first and second portions in the case of a heterozygote. An embodiment of this method is shown in Fig. 4.

Example V.

In this method, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and the reaction is then contacted with the second member of the first specific binding pair, immobilized on a first solid support.

The material not bound to the first solid support is denatured and contacted with an oligonucleotide complementary to a sequence in the strand of the PCR product containing the detectable label. The sequence is between the polymorphic restriction site and the sequence corresponding to the second primer, and the oligonucleotide is tagged with the first member of a second specific binding pair. The reaction is then contacted with the second member of the second specific binding pair, immobilized on a second solid support, and the ratio of the level of the detectable label bound to the first solid support compared to the level of the detectable label bound to the second solid support is determined. A ratio of 0:1 is an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:0 is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:1 is an indication of a heterozygote. These ratios are correct in cases where the total amount of the material not bound to the first solid support is used in the following steps, and should be adjusted accordingly, if a different amount of the material is used. An embodiment of this method is shown in Fig. 5.

Example VI.

In this method, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and a first oligonucleotide tagged with the first member of a first specific binding pair is annealed and ligated to the single-stranded ends generated in the digestion reaction.
The reaction is then contacted with the second member of the first specific binding pair, immobilized on a first solid support.

The material not bound to the first solid support is denatured, and contacted with a second oligonucleotide complementary to a sequence in the strand of the PCR product containing the detectable label, the sequence being between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer. The second oligonucleotide is tagged with the first member of a second specific binding pair. The reaction is then contacted with the second member of the second specific binding pair, immobilized on a second solid support, and the ratio of the level of the detectable label bound to the first solid support compared to the level of the detectable label bound to the second solid support is determined. A ratio of 1:0 is an indication of a homozygote containing the polymorphic restriction site, a ratio of 0:1 is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:1 is an indication of a heterozygote. These ratios are correct in cases where the total amount of the material not bound to the first solid support is used in the following

steps, and should be adjusted accordingly, if a different amount of the material is used. An embodiment of this method is shown in Fig. 6.

Example VII.

In this method, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with the first member of a first specific binding pair, the second primer being tagged with a detectable label. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and contacted with the second member of the first specific binding pair, immobilized on a first solid support.

The material not bound to the first solid support is denatured and contacted with an oligonucleotide complementary to a sequence in the strand of the PCR product containing the detectable label. The sequence is between the polymorphic restriction site and the sequence corresponding to the second primer, and the oligonucleotide is immobilized on a second solid support (e.g., a nylon or nitrocellulose membrane).

The ratio of the level of detectable label bound to the first solid support to the level of detectable label bound to the second solid support is then determined. A ratio of 0:1 is an indication of a homozygote containing the polymorphic restriction site, a ratio of 1:0 is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:1 is an indication of a heterozygote. These ratios are correct in cases where the total amount of the material not bound to the first solid support is used in the following steps, and should be adjusted accordingly, if a different

amount of the material is used. An embodiment of this method is shown in Fig. 5.

Example VIII.

In this method, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site, the first primer being tagged with a detectable label, the second primer being unlabeled. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and a first oligonucleotide tagged with the first member of a first specific binding pair is annealed and ligated to the single-stranded ends generated in the digestion reaction. The reaction is contacted with the second member of the first specific binding pair, immobilized on a first solid support. The material not bound to the first solid support is denatured, and contacted with a second oligonucleotide complementary to a sequence in the strand of the PCR product containing the detectable label. The sequence is between the polymorphic restriction site and either the sequence corresponding to the first primer or the sequence complementary to the second primer, and the second oligonucleotide is immobilized on a second solid support (e.g., a nylon or nitrocellulose membrane).

The ratio of the level of the detectable label bound to the first solid support to the level of the detectable label bound to the second solid support is then determined. A ratio of 1:0 is an indication of a homozygote containing the polymorphic restriction site, a ratio of 0:1 is an indication of a homozygote lacking the polymorphic restriction site, and a ratio of 1:1 is an indication of a heterozygote. These ratios are correct in cases where the total amount of the material not bound to the first solid support is used in the following

steps, and should be adjusted accordingly, if a different amount of the material is used. An embodiment of this method is shown in Fig. 6.

PCR primers containing nucleic acid tags on their 5' ends can also be used in the methods of the invention. These primers can be used in pairs, or in combination with un-tagged primers, in the initial cycles of PCR, followed by the addition of a "universal primer(s)" complementary to the nucleic acid tags in the first primers, and contain detectable labels (e.g., biotin, fluorescent, or ELISA tags). The use of nucleic acid tagged primers in the early rounds of PCR is a cost-effective measure, as only one set of primers, the universal primers, which can be used in the analysis of many different polymorphic sites, need to be detectably labeled. The sets of primers specific for individual polymorphic restriction sites do not have to be tagged with detectable labels, but rather need only to be complementary to the universal primers in their 5' ends.

Example IX.

In another method of the invention, the nucleic acid is amplified by PCR using a first and a second primer flanking the polymorphic restriction site. The PCR product is digested with the restriction endonuclease corresponding to the polymorphic restriction site, and, as shown in Fig. 7, the digestion products are run on a gel (preferably an agarose gel). To simplify the gel reading, the first and second primers are preferably designed to generate a PCR product that is easily resolvable on an agarose gel (that is, preferably larger than 100 base pairs and smaller than 1000 base pairs), and the polymorphic restriction site is preferably

located at an asymmetric position within the amplified fragment. Using this technique, short gel runs can be used for analysis, and the cleaved products easily detected. In the particular example shown in Fig. 8, primers are designed to produce PCR amplified products of 300 base pairs, and cleavage at the RFLP site yields products of 200 base pairs and 100 base pairs.

In a preferred method of carrying out this method, sets of primer pairs are provided which detect a number of RFLP markers. Each set of primers, for example, may be provided in one of the wells of a 96-well microtiter plate, and PCR reactions run independently. Following restriction digestion, the reaction products are transferred to an agarose gel and separated by electrophoresis. A typical result of this method is shown in Fig. 8.

Detection of the amplified and cleaved products after electrophoretic separation may be carried out by standard methods of DNA staining (e.g., ethidium bromide staining) or blotting (e.g., Southern blotting). Alternatively, one or both of the PCR primers may be detectably labeled, and the labels detected as described above.

Example X.

A preferred use of the methods of the invention is in conjunction with a method called RFLP subtraction. RFLP subtraction provides a large number of polymorphic genetic markers, while the methods of the present invention provide efficient methods for their analysis.

Carrying out RFLP subtraction results in the purification of fragments that are present in one population (the tracer) but absent in another (the driver). Purification is achieved by removing all of the fragments in the tracer DNA that have counterparts in the

driver DNA using subtractive hybridization (Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, Harcourt Brace Javanovich, New York, 1990). In RFLP subtraction, the tracer is a size fraction of digested DNA from one strain and the driver is the same size fraction from a polymorphic strain. The products obtained after removing the common sequences are RFLPs; they are sized tracer fragments whose driver counterparts are not found in the same size fraction.

There are three steps in RFLP subtraction: preparation of the driver and tracer, subtractive hybridization, and removal of non-hybridizing sequences from the tracer. To prepare the driver and tracer DNA, genomic DNA from two different strains is digested with a restriction endonuclease, and the ends of the restriction fragments from each strain are capped with different oligonucleotide adapters. The low molecular weight fragments are then purified from a slice of an agarose gel and amplified using one of the adapter strands as a PCR primer. A biotinylated primer can be used to amplify the driver so that driver DNA can be removed following the subtractive hybridizations by binding to avidin coated beads.

Three rounds of subtractive hybridization are performed to remove tracer sequences that also occur in the driver. A small amount of tracer is mixed with an excess of biotinylated driver, the mixture is denatured and allowed to re-anneal. Most tracer sequences will hybridize to complementary biotinylated driver strands. Some tracer sequences, however, are not represented in the driver because they reside on large restriction fragments (i.e., they are RFLPs) or are missing from the driver genome. These fragments will have no complementary biotinylated strands with which to anneal. The biotinylated driver DNA, and any tracer that has

annealed to it, is then removed using avidin-coated beads. The unbound fraction is then subjected to two more rounds of subtractive hybridization, tracer DNA remaining after the third round is amplified, and poorly hybridizing sequences are removed.

Example XI.

Figure 9 shows a preferred method for cloning polymorphic restriction fragments. The object of this method is to clone restriction fragments from organism B (generated by restriction endonuclease A) that do not contain cleavage sites for restriction endonuclease B, and which correspond to restriction fragments in organism A (generated by restriction endonuclease A) that do contain at least one restriction site for restriction endonuclease B. These polymorphic restriction fragments are useful as CAPS markers for the detection methods described above.

Referring to the method outlined in Fig. 9, in step A, genomic DNA isolated from polymorphic individuals A and B is separately digested with restriction enzyme A, which preferably leaves so-called sticky ends. An oligonucleotide adaptor (#1), with complementary sticky ends, is ligated to the restriction fragments from individual A. A different oligonucleotide adaptor (#3) is ligated to the restriction fragments from individual B.

In step B, the restriction fragments from step A are cleaved with restriction endonuclease B, which again preferably leaves sticky ends. In the case of the DNA fragments from individual A, an oligonucleotide adaptor (#2), with complementary sticky ends for enzyme B, is ligated to the restriction fragments generated by cleavage with enzyme B.

In step C, the DNA fragments from individual A are amplified using the polymerase chain reaction (PCR) with an oligonucleotide primer complementary to adaptor #1 and with a biotinylated oligonucleotide primer complementary to adaptor #2.

In step D, the amplified products originating from individual A are mixed with the non-amplified fragments of step B from individual B. The mixed DNA fragments are then heat denatured, annealed, and adsorbed onto an avidin-coated solid support (e.g., beads). The avidin coated support containing the adsorbed fragments is thoroughly washed. If desired, the adsorbed fragments may be eluted, re-amplified with the same primers as above, adsorbed onto a fresh avidin-containing support, and thoroughly washed. This step can be repeated as many times as is necessary or desired.

In step E, the fragments adsorbed to the avidin-coated beads are eluted and amplified using PCR with primers complementary to adaptor #3. The amplified products should correspond to the desired restriction fragments described above. These amplified fragments are cloned and then tested individually using the Southern DNA blot hybridization method for their ability to display the desired RFLP.

Other Embodiments

The above examples are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing form the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. All publications cited herein are

fully incorporated by reference herein in their entirety. Other embodiments are in the claims set forth below.

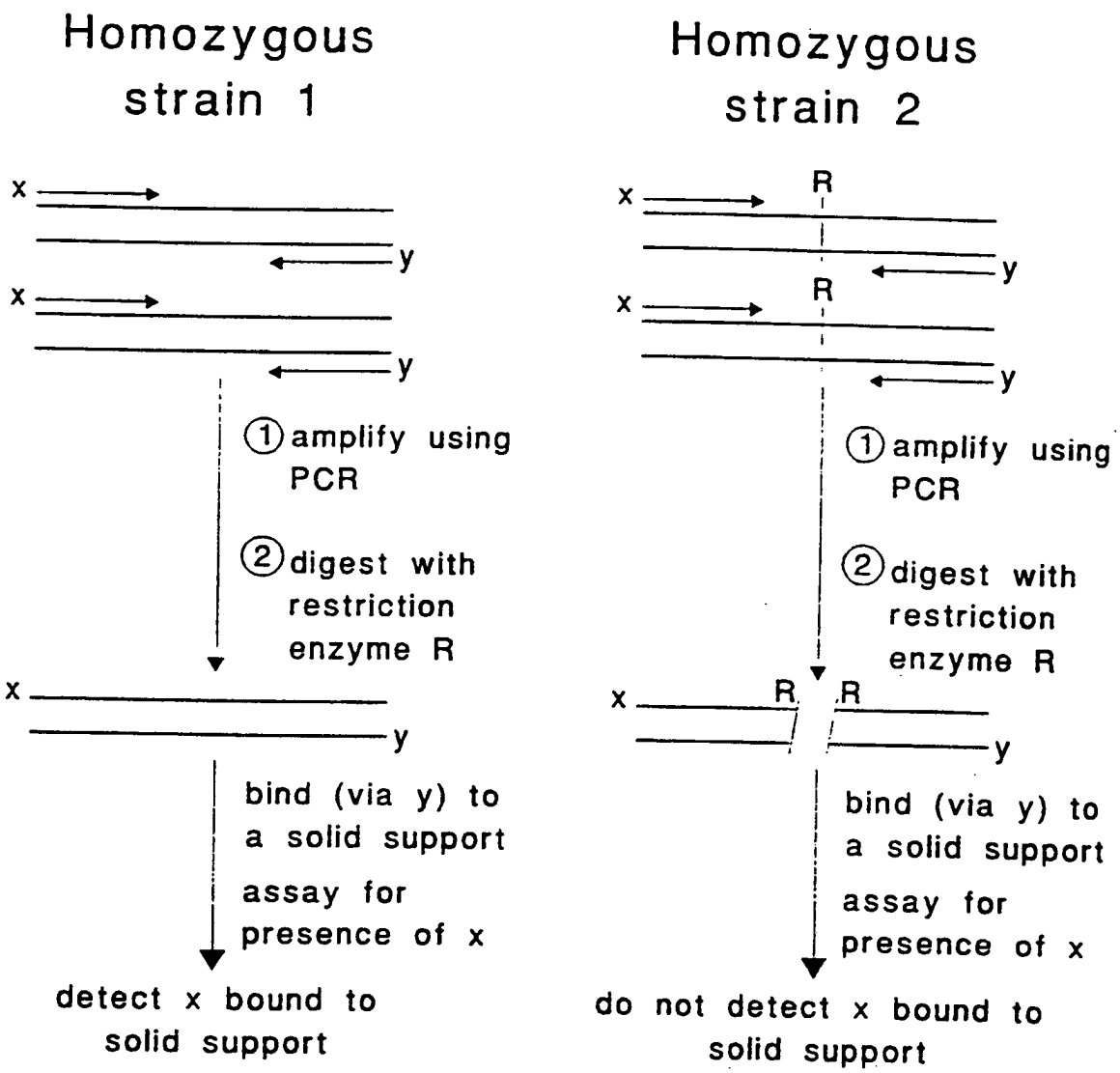
Homozygous strain 1
x
y
x
y
① amplify using PCR
② digest with restriction enzyme R
x
y
bind (via y) to a solid support
assay for presence of x
detect x bound to solid support
Homozygous strain 2
x
R
y
x
R
y
① amplify using PCR
② digest with restriction enzyme R
x
R
R
y
bind (via y) to a solid support
assay for presence of x
do not detect x bound to solid support

FIG. 1

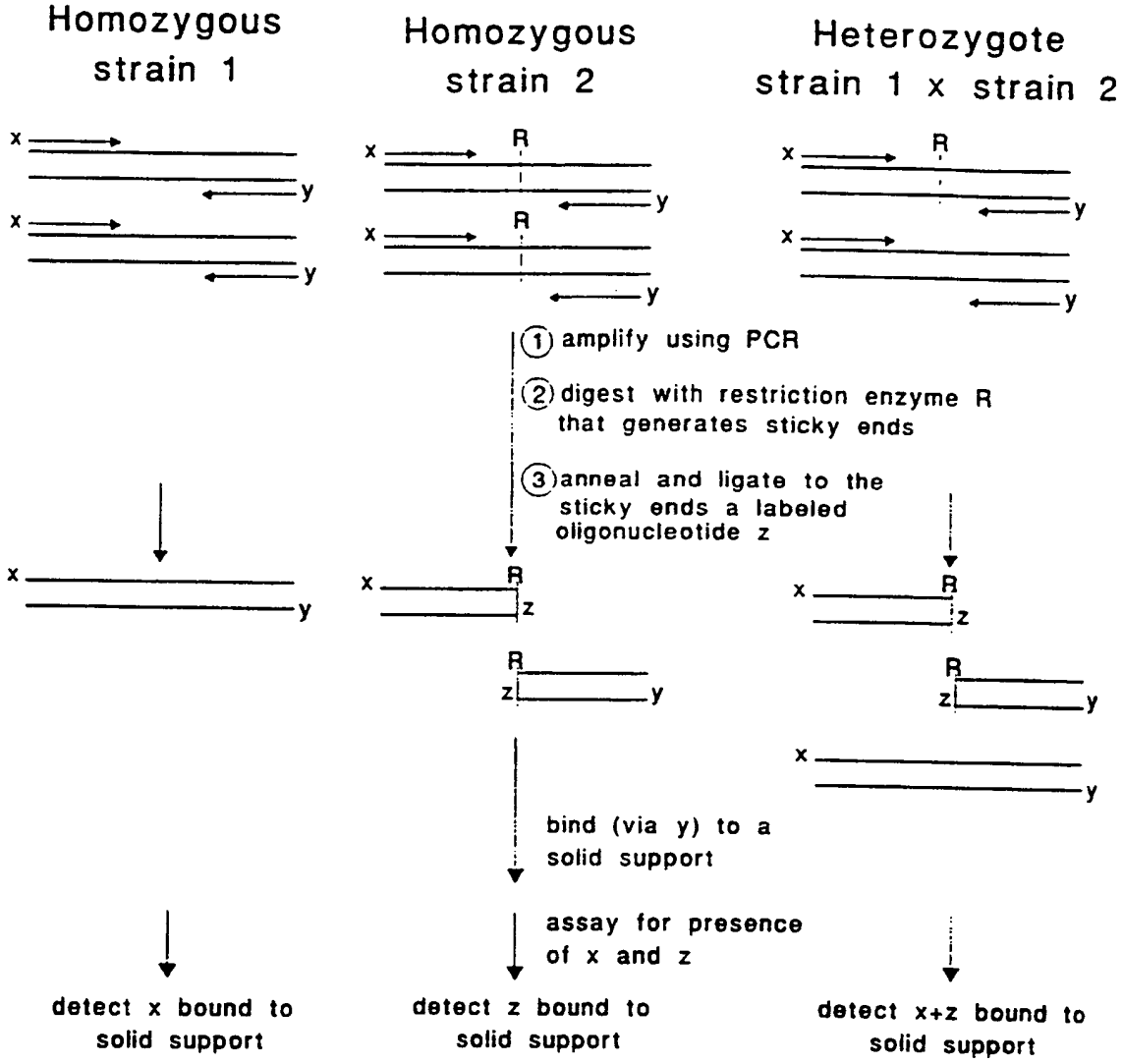
Homozygous strain 1
Homozygous strain 2
Heterozygote strain 1 x strain 2
x
y
R
z
① amplify using PCR
② digest with restriction enzyme R that generates sticky ends
③ anneal and ligate to the sticky ends a labeled oligonucleotide z
bind (via y) to a solid support
assay for presence of x and z
detect x bound to solid support
detect z bound to solid support
detect x+z bound to solid support

FIG. 2

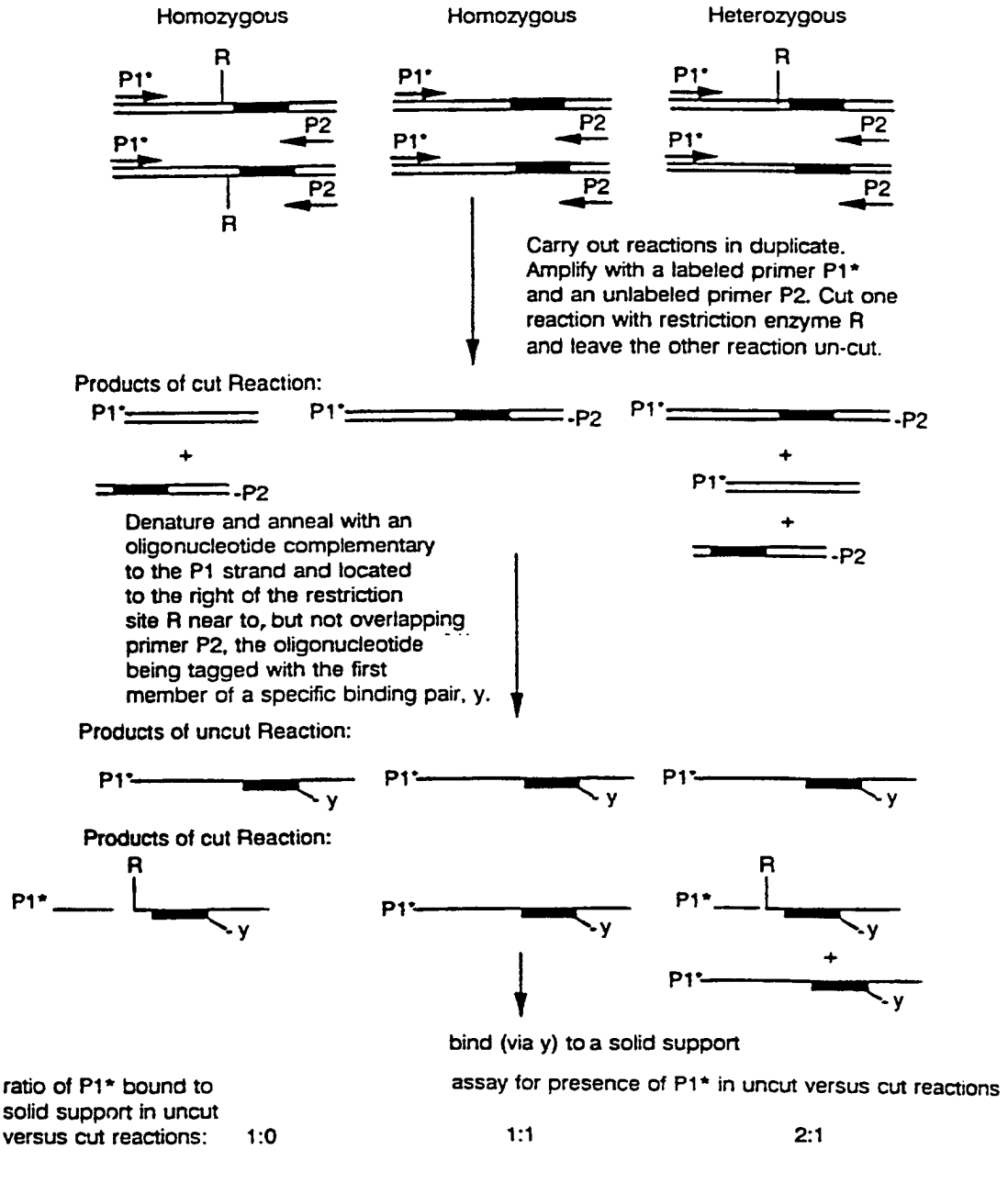
Homozygous
Homozygous
Heterozygous
P1*
R
P2
Carry out reactions in duplicate. Amplify with a labeled primer P1* and an unlabeled primer P2. Cut one reaction with restriction enzyme R and leave the other reaction un-cut.
Products of cut Reaction:
Denature and anneal with an oligonucleotide complementary to the P1 strand and located to the right of the restriction site R near to, but not overlapping primer P2, the oligonucleotide being tagged with the first member of a specific binding pair, y.
Products of uncut Reaction:
Products of cut Reaction:
bind (via y) to a solid support
ratio of P1* bound to solid support in uncut versus cut reactions: 1:0
assay for presence of P1* in uncut versus cut reactions
1:1
2:1

FIG. 3

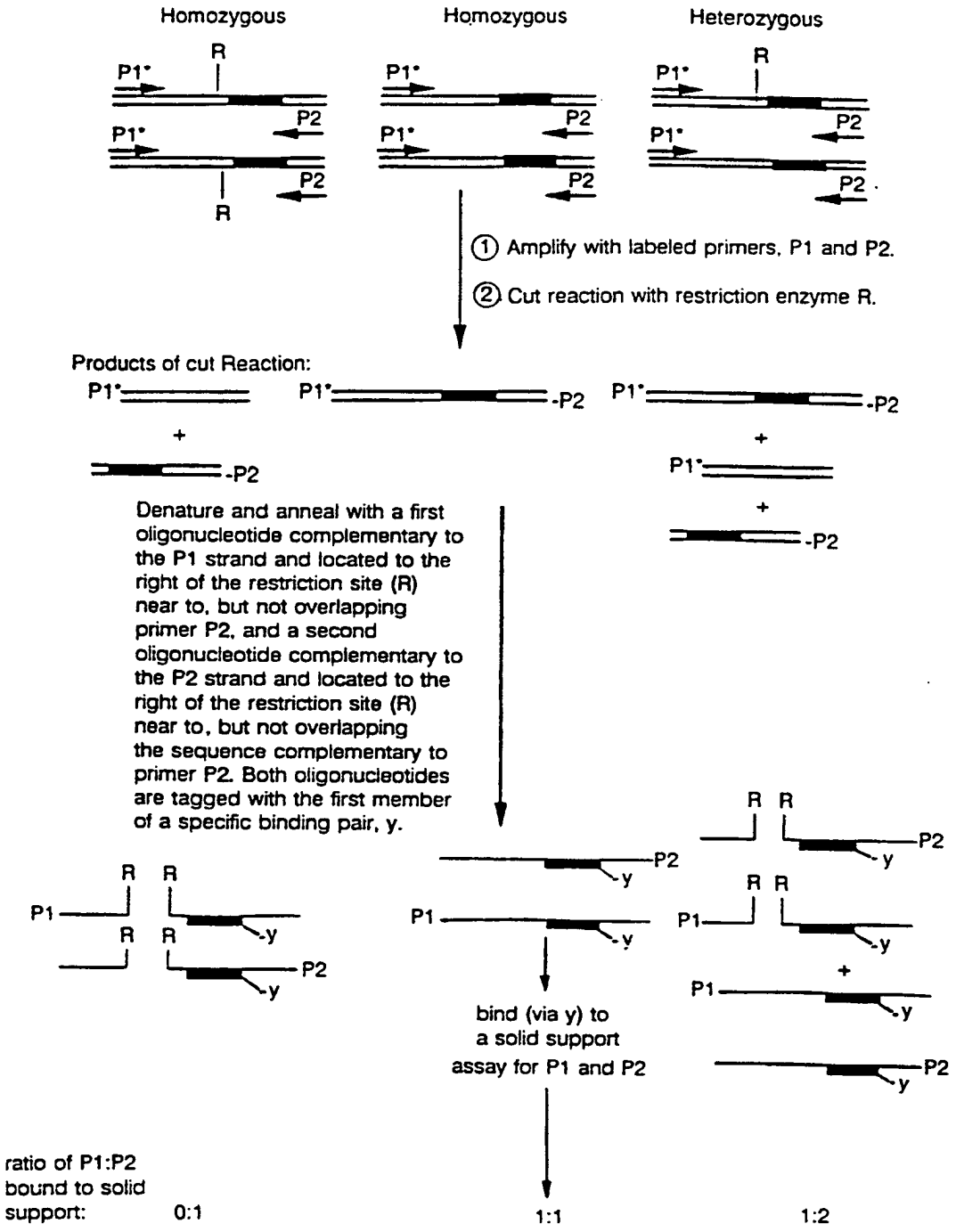
Homozygous
Homozygous
Heterozygous
R
P1*
P2
① Amplify with labeled primers, P1 and P2.
② Cut reaction with restriction enzyme R.
Products of cut Reaction:
Denature and anneal with a first oligonucleotide complementary to the P1 strand and located to the right of the restriction site (R) near to, but not overlapping primer P2, and a second oligonucleotide complementary to the P2 strand and located to the right of the restriction site (R) near to, but not overlapping the sequence complementary to primer P2. Both oligonucleotides are tagged with the first member of a specific binding pair, y.
bind (via y) to a solid support assay for P1 and P2
ratio of P1:P2 bound to solid support:
0:1
1:1
1:2

FIG. 4

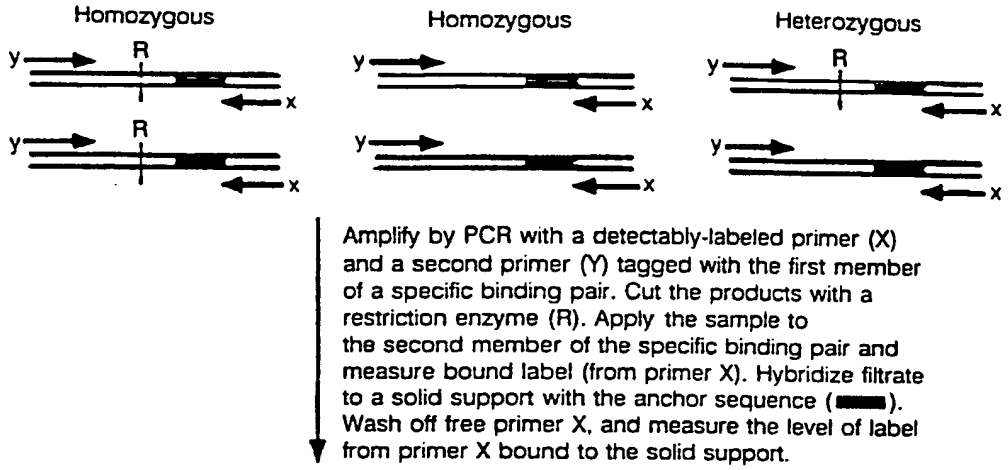
Homozygous
Homozygous
Heterozygous
y
R
x
Amplify by PCR with a detectably-labeled primer (X) and a second primer (Y) tagged with the first member of a specific binding pair. Cut the products with a restriction enzyme (R). Apply the sample to the second member of the specific binding pair and measure bound label (from primer X). Hybridize filtrate to a solid support with the anchor sequence ( ). Wash off free primer X, and measure the level of label from primer X bound to the solid support.
Material bound to second member of specific binding pair
Homozygous
Homozygous
Heterozygous
Material bound to anchor sequence
Homozygous
Homozygous
Heterozygous

FIG. 5

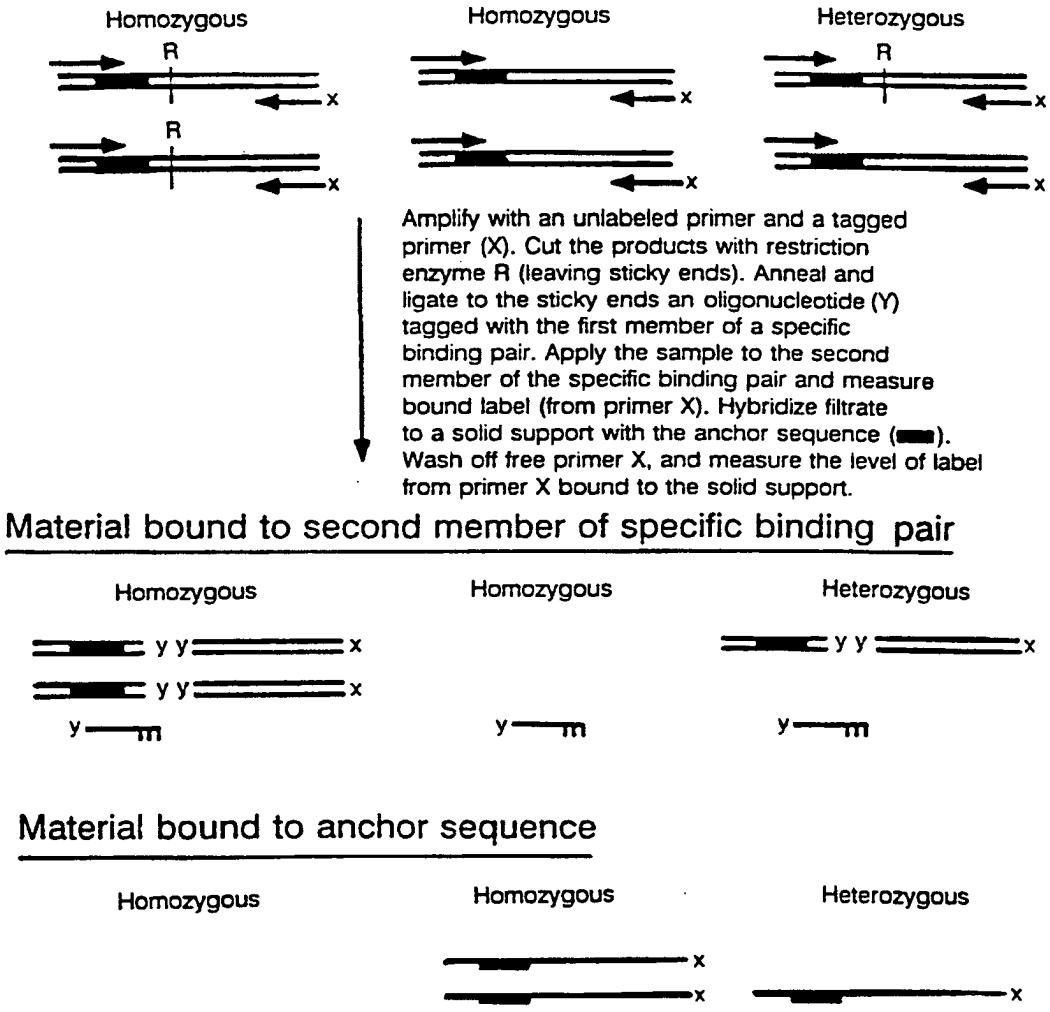
Homozygous
Homozygous
Heterozygous
R
R
x
Amplify with an unlabeled primer and a tagged primer (X). Cut the products with restriction enzyme R (leaving sticky ends). Anneal and ligate to the sticky ends an oligonucleotide (Y) tagged with the first member of a specific binding pair. Apply the sample to the second member of the specific binding pair and measure bound label (from primer X). Hybridize filtrate to a solid support with the anchor sequence (▬). Wash off free primer X, and measure the level of label from primer X bound to the solid support.
Material bound to second member of specific binding pair
Homozygous
Homozygous
Heterozygous
y y
x
y
m
Material bound to anchor sequence
Homozygous
Homozygous
Heterozygous

FIG. 6

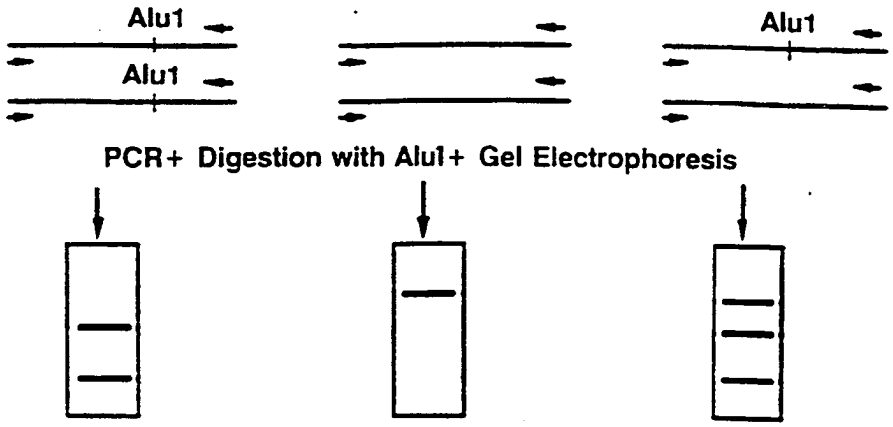
Alu1
Alu1
Alu1
PCR+ Digestion with Alu1+ Gel Electrophoresis

FIG. 7

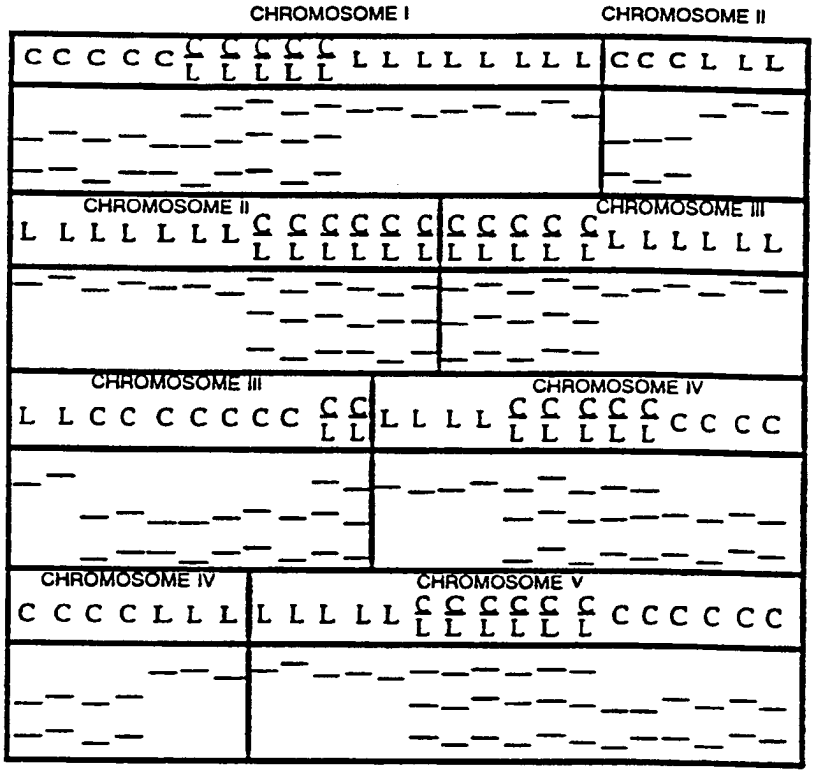
CHROMOSOME I
CHROMOSOME II
CHROMOSOME II
CHROMOSOME III
CHROMOSOME III
CHROMOSOME IV
CHROMOSOME IV
CHROMOSOME V

FIG. 8

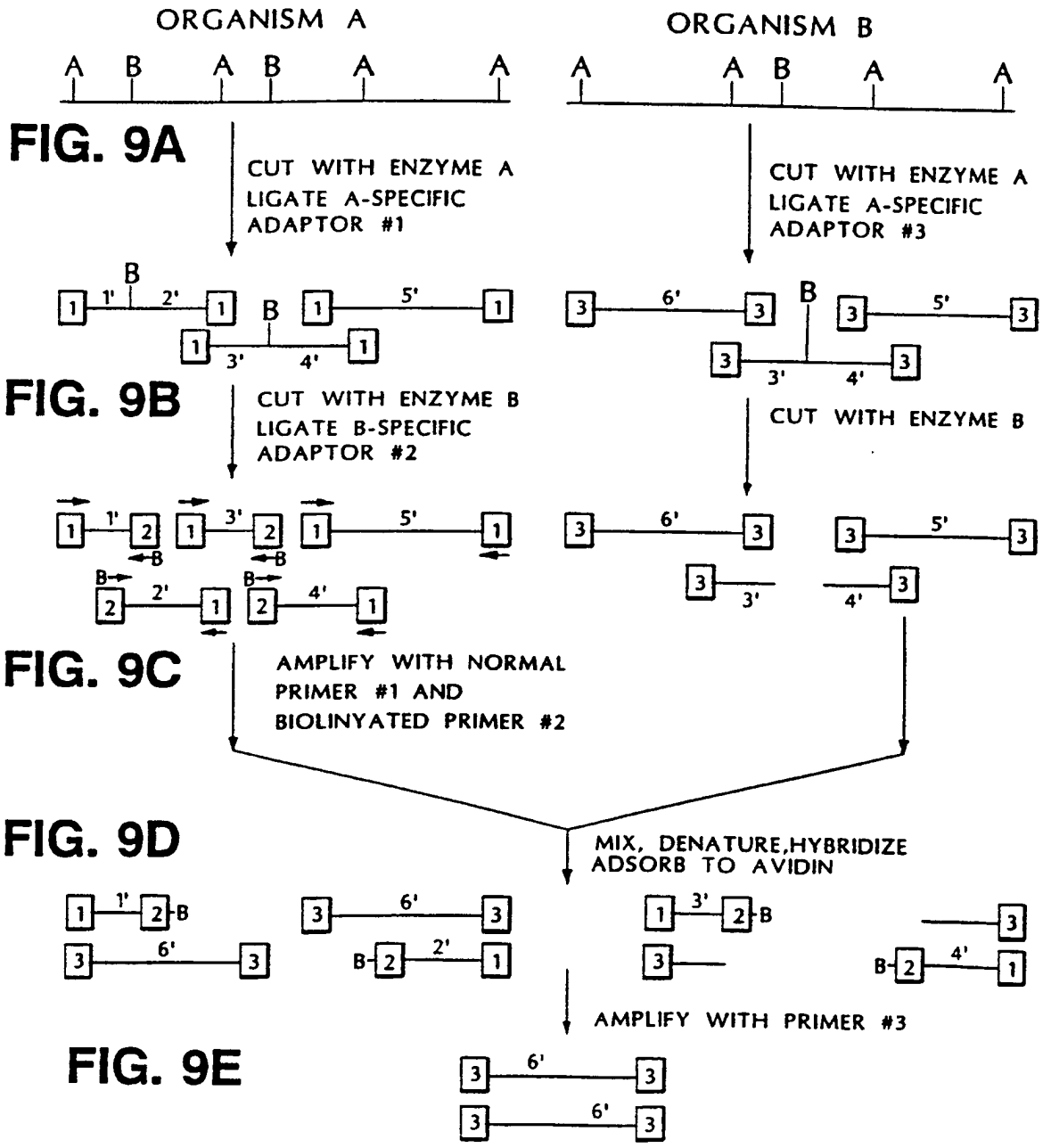

FIG. 9A

FIG. 9B

FIG. 9C

FIG. 9D

FIG. 9E